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Studies on Secondary metabolites of Som(Persea bombycina Kost),a primary host plant of Muga silkworm (Antheraea assamensis Helfer)

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Abstract: The host plant selection behaviour or feeding preferences of the insects are largely mediated by the presence and distribution of secondary metabolites in plants. Quantitative estimation of few secondary metabolites and HPLC analysis of phenolic compounds were carried out from the leaves of Som plant (*Persea bombycina* Kost) which is a primary metabolite of the shimmering yellow golden silk yarn producer, the muga silkworm (*Antheraea assamensis* Helfer). β –sitosterol content in the tender leaves was significantly the highest (1.06%) over semi-mature or mature leaves, whereas Chlorogenic acid content was more in medium leaves (2.06%). Phytic acid content was significantly higher in mature leaves (2310.05 mg/100g), total phenol in tender leaves (1.946%), ADF in mature leaves (26.50%), ADL in mature leaves and tannin in tender leaves (6.71%). Purified phenolic extract when subjected to HPLC analysis, 6 prominent peaks were observed and screening with standard indicates Peaks 1, 2, 3 and 6 correspond to Chlorogenic acid, catechol, Morin and gallic acid, respectively.
Keywords: Som, *Persea bombycina,* Muga silkworm, *Antheraea assamensis*, secondary metabolites, HPLC analysis.

Introduction and Experimental

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development, or reproduction of organisms. Unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survivability, fecundity, or aesthetics, or perhaps in no significant change at all. Secondary metabolites are often restricted to a narrow set of species within a phylogenetic group. Secondary metabolites often play an important role in plant defense against herbivores and other interspecies defenses. Secondary metabolites are chemicals produced by plants for which no role has yet been found in growth, photosynthesis, reproduction, or other "primary" functions. These chemicals are extremely diverse; many thousands have been identified in several major classes. Secondary

metabolites play a major role in the adaptation of plants to the changing environment and in overcoming stress constraints and secondary metabolites through their diversity of functions can be involved in the non-enzymatic plant defense strategy[4]. The apparent lack of primary function in the plant, combined with the observation that many secondary metabolites have specific negative impacts on other organisms such as herbivores and pathogens**,** leads to the hypothesis that they have evolved because of their protective value.

Estimation, importance and biological relevance of Secondary metabolites on the cocoon production of mulberry silkworm, *Bombyx mori* is well documented. Sterol compound, β-sitosterol and β -d-glycoside of β -sitosterol present in mulberry leaf plays the role of a biting factor[7,10]. Terpene compounds, terpinyl acetate, linalyl acetate, linolool and citral act as attractants for *Bombyx mori* [10]. One phenolic acid, cholrogenic acid, is reported to have strong growth promoting action and a role in moulting of B. mori larvae. This acid is indispensable for normal growth of the silkworm on a synthetic diet. This acid could not be replaced by phenyl alanine, tyrosine or digydroxy –phyl-alanine, which has commonly been proposed as preserver of poly phenols.

The biological relevance of host plant derived terpinoids in the cocoons of the tropical tasar silkworm *Antheraea mylitta*[2]. The β-sitosterol in *Moringa oleifera* may be responsible for hypolipidemic, and as well as antioxidant properties[21].
Phenols comprise the largest group of plant secondary metabolites found in both edible and non-edible plants. These compounds have been represented to have multiple biological effects including antioxidant property.

Muga silkworm (*Antheraea assamensis,* Helfer), an economically important insect is unique and prerogative to the North Eastern region of India particularly in the Assam province. The golden yellow yarn produced by this insect is lustrous, highly durable, strongest and toughest of all natural silks having multifarious utilities for which its demand is increasing in world fabric market. The silkworm is polyphagous, multivoltine (5-6 crops per year), semi-domesticated in nature, the worms are reared outdoor on standing trees. On maturity, the worms crawl down at the end of completion of five larval instars, are collected by rearers, and allowed to spin cocoons inside rearing house. Muga silkworm feeds primarily on "Som" (*Persea bombycina* King ex. Kost.) and "Soalu" (*Litsea monopetela* Pers.). The other food plants include "Diglotti" (*Litsea salicifolia* Roxb. Ex. Wall.) and "Mejankori" (*Litsea citrata* Blume) are of secondary importance.

Phytophagous insects show varying degree of association with a particular plant species or group of plants, on which they feed and these plants are referred to as host plants (food plant range) [25]. That the host plant selection behaviour or feeding preferences of the insects are largely mediated by the presence and distribution of secondary metabolites in plant is largely accepted[6,14]. Flavonoids and related phenolic compounds act as strong feeding deterrents to many insects, they may sometimes be stimulatory to other[20, 17,24,8.]

Literature on the leaf biochemical composition of *Persea bombycina* and *Litsea monopetela*, the two primary host plants of muga silkworm, *A. assamensis*, particularly, the secondary metabolite is very meager. In this paper, composition of few secondary metabolites, viz. β-sitosterol, total phenol, Chlorogenic acid, lignins, tannin content in the leaves of these two plants have been described.

Preparation of leaf samples
Leaf samples of three different types (tender, medium and mature) of *Persea bombycina* Kost were collected separately from the two host plants and were properly cleaned and then dried in hot air oven at a temperature ranging between 80-90ºC for several hours till drying completely. The dried leaves were ground in an electric grinder (Make BAJAJ Mixer grinder Gx7) to make it powder. The powdered leaf samples were kept separately in polypropylene containers which were subsequently used for analysis.

Quantification of ß-sitosterol in the leaf sample[12]
Preparation of standard stock solution
10mg of ß-sitosterol working standard was accurately weighted and transferred into a 10mL volumetric flask and dissolved in a mixture of methanol and chloroform (1:1). The volume was made up to the mark using the same solvent to give a concentration of 1mg/mL. The solution was labeled and stored in a refrigerator below 8^0C. The above solution was suitably diluted for further analysis.

Preparation of chloroform alcoholic extract
10g of the leaf sample was taken in a round bottom flask of Soxhlet apparatus and it was dissolved in a mixture of chloroform and methanol (10:1). The solution was refluxed on heating mantle at 95^0C for 2 hours. After completion of refluxing, the syrupy material was centrifuged at 1000RPM for 5minutes.

Collected the extract in a labeled test tube and stored in a refrigerator below 8^0C for future analysis.

Preparation of sample solution
5 ml of the extract was taken in test tubes which were evaporated in a boiling water bath. Then it was cooled and 2 ml of dried acetic anhydride was added followed by 2 drops of concentrated sulphuric acid. The tubes were kept standing for thirty minutes. Absorbance was measured at 655nm against a reagent blank in a spectrophotometer (PC based UV-Vis spectrophotometer (Make: Systronics, Model: 2202). Sterol values were calculated by referring to the standard curve prepared with known concentration of ß-sitosterol (table 1).

Estimation of total phenol content[15]
Preparation of hydroalcoholic extract
1g of leaf sample was weighed and extraction was done with 10mL 80% ethanol thrice taking 5mL each time in a pestle and mortar. Grinded material was centrifuged at 1000RPM for 10 minutes. Collected the extract and pooled in a 500mL beaker and evaporated to dryness which was then redissolved with 5mL of water. The diluted extract was collected in a labeled test tube and stored in refrigerator below 8^0C for future analysis.

Preparation of sample solution
2mL of the extract was taken in test tubes and volume was equalized to 3mL with water. 0.5mL of Folin-Ciocalteau reagent was added to each tube. After 3 minutes, 2mL of 20% Sodium carbonate solution was added to each tube and then kept in boiling water bath for exactly one minute. The tubes were then cooled and the intensity of colour was read at 650nm. The total phenol content (Table 1) was estimated from a standard curve prepared with known concentration of catechol and was expressed as mg phenols per 100g leaf on fresh weight basis.

Quantification of Phytic acid content[27]
Preparation of acidic extract
1g of leaf sample of *Persea bombycina* was taken in a 125mL Erlenmeyer flask and extracted with 50mL of 3% trichloro acetic acid (TCA) for 30 minutes. The suspension was then centrifuged and transferred a 10 ml aliquot of the supernatant to a 40mL conical centrifuge tube. 4mL of FeCl3 solution was then added rapidly and heated in a boiling water bath for 45 minutes which was again centrifuged and supernatant was decanted. The precipitate was washed twice by dispersing well in 20mL 3% TCA, heated in boiling water for 10 minutes and centrifuged. The preceipitate was again dispersed in few ml of water and 3mL 1.5N NaOH is added. The volume was then made up to 30mL with water and heated in boiling water for 30 minutes, which was then filtered hot through Whatman No. 2 filter paper. The precipitate was again washed with 60-70mL hot water and filtrate was discarded. The precipitate was dissolved from the filter paper with 40mL hot 3.2N HNO3 into a 100mL volumetric flask. The paper was washed with several portions of water, collecting and washing in the same flask. The content was stored in cooled room temperature.

Preparation of sample solution
5mL of acidic extract was aliquot to 100mL volumetric flask and diluted to approximately 70 mL. Then 20mL 1.5M potassium thiocyanade was added, volume made up to 100mL and color intensity was read within one minute bat 480nm. A reagent blank was also run with each set of samples. A standard curve was prepared with Fe(NO3)3 from which phytate was calculated by the equation as follows:

Phytate *P* mg/100g sample= (µg Fe x 15)/weight of sample (g)

Estimation of total Chlorogenic acid content[18]
Preparation of hydroalcoholic acidic extract
5g of the leaf sample was taken in a round bottom flask and refluxed twice with 125mL 80% ethanol (adjusted pH 4.0 with 2.5 N HCl) for 30 minutes. The refluxed solution was centrifuged at 700RPM for 5 minutes. About 250 ml of the extract was collected and stored in a cool place.

Preparation of sample solution
5mL of the extract was taken in test tubes which were dried in a vacuum oven at 50ºC and 700mm pressure for 2 hours. The dried extract was dissolved in 4.75mL acetone to which 0.25mL Titanium Chloride was added. Absorbance was measured at 450nm against a reagent blank and calculated by referring to the standard curve prepared with known concentration of Chlorogenicio acid (Table 1).

Estimation of total Acid Detergent Fibre and lignin content[1]
Preparation of Acid Detergent extract
1g of the leaf sample was taken in a round bottom flask with 100mL of acid detergent solution (20g of cetyl methyl ammonium bromide in one litre of 1N sulphuric acid). Heated to boil for 5 to 10 minutes and the heat is reduced to avoid foaming as boiling begins. The material was refluxed for 1 hour after the onset of boiling adjusting boiling to slow, even level. The container was then removed, swirled and the content was filtered through a preweighed sintered glass

crucible (G-2) by suction and washed with hot water twice. The filtrate was then washed till it becomes colourless and dried at 100ºC for overnight. Collected the fibers and stored in cooled desiccators.

Preparation of fiber sample

The cooled fiber materials were took weight and this is the ADF (acidic detergent fiber) expressed in percentage i.e. W/Sx100, where W is the weight of the fibre and S is the weight of the sample.

Preparation of Lignin sample

The ADF obtained from the previous method was transferred to a 100mL beaker with 25-50 mL of 72% sulphuric acid to which one gram asbestos was added. It was then allowed to stand for 3 hours with intermittent stirring with a glass rod. The acid was then diluted with water and filtered with preweighed Whatman No 1 filter paper. The glass rod and the residue were washed several times to get rid of acids. The filter paper was then dried at 100ºC and weight was taken after cooling in desiccators. The filter paper was transferred to a preweighed silica crucible and it was ashed with the content in a muffle furnace (INSIF Make) at 550 ºC for about 3 hours. The crucible was cooled in a desiccator and weighed. For blank, 1 gram asbestos was taken in a similar manner.

ADL content was calculated using the following equation.

$$\% \text{ ADL} = \frac{\text{Weight of 72\% } H_2SO_4 \text{ Washed fibre} - \text{Ash (Test-Asbestos blank)}}{\text{Weight of sample}} \times \frac{\text{(Test-Asbestos blank)} \times 100}{}$$

Estimation of total tannin content[22]

Preparation of tannin extract

0.5g of powdered leaf sample was weighed taken in a 250mL conical flask and 75mL distilled water was added to it. The flask was gently heated and boiled for 30 minutes. The solution was then centrifuged in a centrifuge (Research Centrifuge, Make: Remi, Model: R-24) at 2000 rpm for 20 minutes and the supernatant was collected in 100mL volumetric flask and volume made up to 100mL. The extract was stored in cool place.

Preparation of sample solution

1mL of the sample extract was transferred to a 100mL volumetric flask containing 75mL water to which 5ml of Folin-Denis reagent, 10mL of sodium carbonate solution and diluted to 100mL with water. It was then shaken well and absorbance was read at 700nm in spectrophotometer after 30 minutes. The tannin content was estimated from a standard curve prepared with known concentration of tannic acid and was expressed as percentage on dry weight basis.

High Performance Liquid Chormatography analysis of phenolic compounds:

Preparation of sample extracts

100g of fresh leaves of *P. bombycina* was crushed in the presence of methanol HCl. After crushing into paste, the content was kept under methanol HCl for 48 hours (<) with intermittent shaking. Then the supernatant was collected after filtration followed by centrifugation (5,000rmp for 10 minutes). The extract was concentrated and dried to a syrupy material which was then treated with 2.0% sodium Hydroxide followed by extraction with 200mL petroleum ether. The organic phase was then discarded and the aqueous phase was again acidified with dilute 2.5N HCl and then extracted with dichloromethane. The extract was collected in a 500mL beaker and evaporated to dry. The dry extract was stored in cool place.

Preparation of sample solution

500µg of extract was dissolved in 30mL of HPLC grade methanol in a 100mL beaker. The solution was filtered 3 times through whatman filter paper. Then filtered 3 times through 0.45µ membrane filter. The filtrate solution was kept in a labeled test tube in cool place.

Various trials were carried out and the following chromatographic conditions were optimized.

Optimized Chromatographic Conditions:

Stationary phase	: …….C_{18} (4.6 x 250mm i.d., 5µm)
Mobile Phase	: (A) 0.5% acetic acid: 95.5% water (B)0.5%acetic acid: 95.5% acetonitrile
Flow rate	: 1mL/min
Injection volume	: …….
Oven temperature	:……..
Model	: 600 Waters 2489
Mode	: UV detector
Wavelength	: 254nm

The experiments were carried out at room temperature of about 20^0C. Both the standard and sample solutions

were injected and experiment carried out using the above optimized chromatographic conditions and the chromatograms (Table 1) of standard and sample solutions were recoded, from which the amount of phenol present in the leaf extract was determined. The phenol compounds were identified by comparing their relative retention times with those of standards mixture chromatogram.

Result and Discussion:

Data on composition of different secondary metabolites in leaves of different maturity levels of *P. bombycina* are presented in Table 1. ß-sitosterol content in the leaves exhibited a decreasing trend with the increase in maturity level, significantly the highest being in tender leaves (1.06 %). This may be one of the reasons why the *A. assamensis* silkworms are attracted most towards the tender leaves of this host plant. First instar muga silkworms were reported to be more attracted towards tender leaves of *P. bombycina*[3] and for this behaviour, higher ß-sitosterol content may be the reason as this compound acts as a biting factor for another silkworm, *B. mori* [9,10].

Table 1. Composition of different secondary metabolites in the leaves of *P. bombycina* of different maturity

Sl. No.	Chemicals	Maturity level			S. Ed. (±)	$CD_{0.05}$
		Tender	Medium	Mature		
1.	ß-sitosterol (%)	1.06 a	0.82 b	0.69 c	0.017	0.034
2.	Chlorogenic acid (%)	1.81 b	2.06 a	1.45 c	0.044	0.089
3.	Phytic acid (mg/100g)	83.33 c	1642.20 b	2310.05 a	224.103	462.55
4.	Total phenol (%)	1.946 a	1.182 b	0.712 c	0.015	0.030
5.	Acid detergent fibre (%)	16.90 c	23.35 b	26.50 a	0.286	0.575
6.	Acid detergent lignin (%)	8.02 c	13.52 b	15.83 a	0.216	0.437
7.	Tannin (%)	6.71a	2.05c	3.00b	0.37	0.77

Table 2. Retention time and area of the phenolic compounds from *P. bombycina leaves.*

Sl. No.	Peak no.	Retention Time	Area	% Area
1	Peak1	2.339	2517660	10.01
2	Peak2	2.547	9256141	36.81
3	Peak3	2.646	3697496	14.71
4	Peak4	2.755	3592798	14.29
5	Peak5	2.963	4800414	19.09
6	Peak6	3.543	1278946	5.09

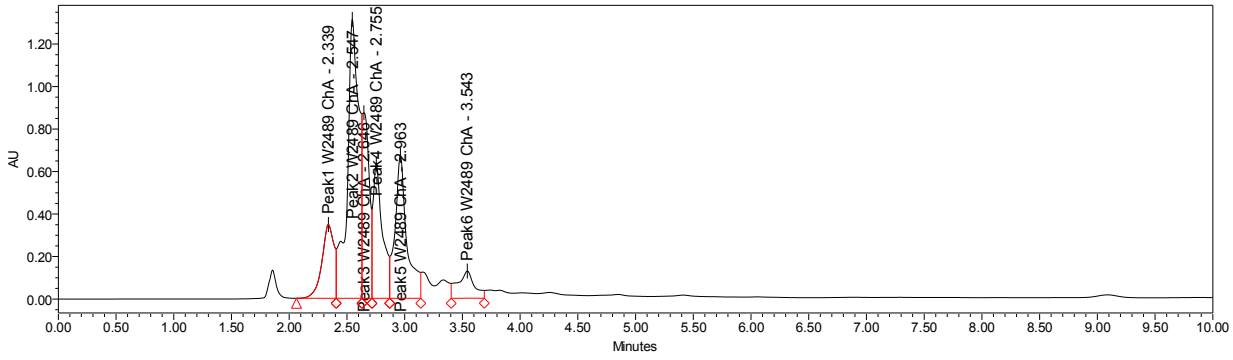

Fig. 1. Chromatogram of the phenolic compounds from *P. bombycina leaves*;
1. Chlorogenic acid, 2. Catechol, 3. Morin and 4. Gallic acid

Phenolic compounds are important in several respects during the development of *Bombyx mori*. Apart from acting as biting factors, morin and chlorogenic acid enhance the rate of development, especially in the early stages isolated chlorogenic acid from mulberry leaves and noted that it is also a "biting factor" of *B. mori*[13,19]. Chlorogenic acid acts as a biting factor for mulberry silkworm *Bombyx mori*. It is indispensable for normal growth of the silkworm on a synthetic diet. Chlorogenic acid could not be replaced by phenylalanine, tyrosine or dihydroxy-phenyl-alanin, which has commonly been proposed as precursors of the polyphenols. Because of this, phenolic compounds were used in the synthetic diet of young larvae and demonstrated the growth promoting action of chlorogenic acid[9]. In the present investigation, chlorogenic acid content in the leaves of *P. bombycina* was more in medium leaves (2.06%) compared to tender or mature leaves.

Phytic acid (myo-inositol hexaphosphate) is a common storage form of phosphorus in seeds and is considered as an antinutritional factor. The complexing of phytic acid with nutritionally essential elements and the possibility of interference with proteolytic digestion have been suggested as responsible for antinutritional acitivity. It also interferes with calcium and iron absorption and phosphorus in phytic acid is not nutritionally available to monogastric animals. Phytic acid constitutes 1-3% of most plant seeds[5]. The content of this acid in *P. bombycina* leaves showed an increasing trend with maturity level being significantly the highest in mature leaves (2310.05 mg/100 g).

Good quality plants (on the basis of rearing performance) showed higher total phenol content, which ranged from 1.98% in least preferred leaves to 6.26% in most preferred genotypes of *P. bombycina*[11]. Total phenol content was found to be significantly more in tender leaves of *P. bombycina* (1.946%) compared to medium (1.182%) or mature leaves (0.712%). Phenols are said to offer resistance to diseases and pests in plants. Age dependent variations in the distribution of chemical defenses within and among plants have bearing on herbivore behaviour and fitness[23]. Higher content of phenols in tender leaves also supports the optimal defence theory, where young and semi-mature leaves having high fitness and high probability of attack tend to have higher concentration of defence metabolites.

Fibre is the ash free material and reduction in the fibre content had been established as an advantage for better silkworm crop yield[28]. Acid Detergent Fibre (ADF) content of *P. bombycina* was more in mature leaves (26.50%) compared to medium (23.35%) or tender leaves (16.90%). Lignins are phenolic polymers present in the cell walls of plants which are responsible together with cellulose, for the stiffness and rigidity of plants stems. It acts as a physical barrier against invading pathogens. Acid Detergent Lignin (ADL) content of *P. bombycina* leaves exhibited an increasing trend with increase in maturity level (Table 1.).

Tannins are secondary metabolites of plants, non-nitrogenous, phenolic in nature and are present in all plant materials. It gives immunity to seeds attack by birds and diseases; they on the other hand display impaired nutritional quality, lower digestibility and reduction of food consumption. In, *P. bombycina* leaves, tannin content was found to be significantly more in tender leaves (6.71%) compared to other types of leaves.

HPLC analysis of phenolics

Purified phenolic extract when subjected to HPLC analysis, 6 prominent peaks were observed. Screening with standard indicates Peaks 1, 2, 3 and 6 correspond to Chlorogenic acid, catechol, Morin and gallic acid, respectively (Fig. 1).

Conclusion

Secondary metabolites play major role in accepting a leaf or diet by several lepidopteron insects including silkworms. Accurate quantitative estimation gives an insight into the composition of such metabolites and may help in developing an artificial diet for the silkworms. Since, *A. assamensis* silkworms are reared outdoor and its domestication under indoor condition requires developing such an effective diet, present study may be useful for including the feeding stimulants in preparing the diet at required quantity.

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